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(54) Title: EXPRESSION SYSTEM

(57) Abstract: A recombinant microorganism comprises an asporogenic Bacillus *subtilis* strain in which a gene encoding a protease enzyme has been downregulated or inactivated. In particular sigma factor*spoIIAC* is inactivated such that the strain is asporogenic. These strains are particularly useful as expression vehicles for proteins such as protective antigen (PA) of *Bacillus anthracis*.

Expression system

The present invention relates to an expression system, useful in the production of a range of proteins, which is derived from *B. subtilis*. In addition, the invention relates to the use of this system in the production of proteins such as antigens used in vaccines, for example recombinant Protective Antigen (PA) of *Bacillus anthracis*.

The protective antigen (PA) of *Bacillus anthracis*, the causative agent of anthrax, is the key component of the current UK human anthrax vaccine (Hambleton, P. et al., (1984) Anthrax: the disease in relation to vaccines. Vaccine 2;125-132). Due to safety concerns relating to the handling of *B. anthracis* attempts have been made to express PA from a variety of different hosts. These include attenuated strains of *B. anthracis* (Belton, F.C. et al., (1954) British Journal of Experimental Pathology, 35;144-152 and Turnbull, P.C.B. (1991) Vaccine **9**,533-539), *B. subtilis* (Ivins, B.E. et al., Infection and Immunity (1986), 54;537-542, Baillie, L.W.J. et al., (1994) Letters in Applied Microbiology 19;225-227, Baillie, L.W.J., et al., (1996) In *Proceedings* of the International Workshop on Anthrax, 19-21 Sept 1995, Winchester, UK. Salisbury Medical Bulletin 1996, 87 (Special Suppl.) 133-135 and Baillie, L.W.J., et al., (1998) Journal of Applied Microbiology **84**;741-746, *B. brevis* (Oh, H-B., et al., (1998) Abstracts of the 3rd International Conference on Anthrax, 7-10the Sept, 1998), *vaccinia* (Iancono-Connors, L.C., et al., (1990) Infect. Immun. 58;366-372), *baculovirus* (Iancono-Connors, L.C., et al., (1990) Infect. Immun. 58;366-372), *Salmonella typhimurium* (Coulson N.M. et al., Vaccine (1994) 12;1395-1401) and *E. coli* (Singh, Y. et al., (1989), J. Biol. Chem., 264;11099-11102 and Vodkin M.H. et al., (1993) Cell, 34;693-697).

While none of these systems is ideal, to date, the best yields have been achieved using *Bacillus* spp. *Bacillus* spp. have a

history of safe use in both food and industry. A number of products from *Bacillus* spp. are generally recognised as safe for specific uses by the U.S. Food and Drug Administration. These products include enzymes for food processing as well as whole foods produced from the microorganism (*B. subtilis natto*).

The PA gene is expressed well in *B. subtilis* achieving levels of expression higher than those obtained with the current *B. anthracis* based system (Ivins, B.E. et al., (1986), supra.). In addition the protein is exported into the culture supernatant, simplifying subsequent purification protocols (Miller, J. et al., (1998) Letters in Applied Microbiology, 26;56-60).

A draw back of using bacilli is their ability to produce degradative proteases (Baillie, L.W.J. et al., (1994) supra.) and resistant spores (Driks, A. (1999) Microbiology and Molecular Biology Reviews **63**;1-20).

Strains of *B. subtilis* have been engineered to overcome the problem of proteolytic degradation of expressed proteins. WB600 is one such a strain (Xu-Chu, Wu et al., (1991) Journal of Bacteriology. 173,4952-4958).

Using this strain a high level laboratory scale, expression and purification system, has been developed for the production of pure rPA. (Miller, J. et al., (1998) Letters in Applied Microbiology, 26;56-60).

In animal studies rPA invoked total protection to aerosol challenge with spores of a vaccine resistant strain of *Bacillus anthracis* (McBride, B.W., et al., (1998) Vaccine **16**;8,810-817).

The ability of the production strain WB600 to form resistant spores raises the possibility of environmental contamination when the strain is grown to large numbers such as is the case

with fermenters. This potential is of particular concern to industry who cannot risk cross-contamination with other products. Therefore, such systems require the use of a dedicated production plant.

Spore formation is regulated by a complex network of genes which function as a cascade (Hoch, J.A. spo0 Genes, the phosphorelay, and the Initiation of sporulation. In *Bacillus* and other Gram-positive bacteria. Editors Sonenshein, A.L. et al., chapter 51;747-755, American Society for Microbiology, Washington D.C.). Targeted inactivation of early sporulation genes, such as spo0A, will result in a strain incapable of forming spores. Sporulation deficient strains in which the early sporulation genes spOA are deficient, may be physiologically compromised. Consequently, the effects of other genetic modifications may not be tolerated and strains may become non-viable.

The applicants have found however that strains of *B. subtilis* can be produced which are asporogenic and also protease deficient.

The present invention provides a recombinant microorganism comprising an asporogenic strain of *Bacillus subtilis* in which at least one gene which encodes a protease enzyme has been downregulated or inactivated.

Strains of this type provide good yields of heterologous proteins which the strain may be engineered to express, and do not generate problems associated with sporulation.

Suitable protease enzyme genes within the *B. subtilis* strain which may be inactivated or downregulated include serine alkaline protease E (subtilisin 1143 bp) (*aprE*), bacillopeptidase F (*bpr*), extracellular serine protease (*epr*) (1935 bp), extracellular metalloprotease (*mpr*) (939 bp),

extracellular neutral protease (*nprB*) (614 bp) and extracellular neutral metalloprotease (*nprE*)(1563 bp).

The sequences of these genes can be determined from the literature (see in particular Kunst et al. Nature (1997) 390:249-256).

Preferably more than one, more preferably at least 3 and most preferably all of these genes are inactivated.

Inactivation or downregulation of the target sequence may be carried out using any of the known methods. For example, the sequence may be partially or totally deleted, and additionally may be subject to allelic replacement, or may be subjected to mutation including insertional mutation in order to inactivate the encoded protease enzyme. Alternative methods of gene inactivation such as the use of sense or antisense RNA constructs in order to prevent transcription of the gene sequence may also be employed.

In particular, the strain is rendered asporogenic by the inactivation of a sequence encoding sigma factor *spoIIAC*. The applicants have found that by inactivating this specific fragment of a stage II sporulation gene, stable asporogenic recombinant strains are produced which are robust and are particularly useful in the production of vaccines.

The spoIIA gene is one of the nine known stage-II loci. Mutation of this gene can block development at the septation stage of sporulation (Losick et al. supra.). This gene has been cloned and sequenced and found to be transcribed as a polycistronic unit of three cistrons encoding proteins of 13, 16 and 29 kd. These are encoded by regions designated *spOIIAA*, *spoIIAB* and *spoIIAC* respectively.

The sequence encoding sigma factor *spoIIAC* is suitably inactivated using the methods described above in relation to the inactivation or downregulation of the protease enzyme genes. Preferably however, the nucleic acid sequence encoding sigma factor *spoIIAC* within the *Bacillus subtilis genome* is partially or totally deleted, and optionally replaced with a different sequence. This different sequence may comprise, for example a marker gene, such as an antibiotic resistance gene, which will assist in the transformation process in selecting transformed clones.

Other regions of the spoIIA gene may also be deleted if desired.

In a particular method, the 5′ and 3′ regions flanking the target sequence to be deleted are amplified from *B. subtilis* chromosomal DNA in two separate reactions. A first amplification reaction (for example a polymerase chain reaction (PCR) reaction) is carried out using 5XBA-5XHO and 3XHO-3XBA primer pairs. The two amplified flanks are then used to self-prime each other and generate the locus minus the target sequence. This truncate can then be amplified by external primers in a second amplification (PCR) reaction.

Alternatively, the two flanking products from the first reaction may be cut with a suitable restriction endonuclease for example *XhoI*, mixed and ligated. Suitably, the primers are designed such that the 5′flank amplicon is up to 700 base pairs in length whilst the 3′ flank is 800 or more base pairs in length. This allows clear identification of the ligation for example using gel chromatography. When the product of the ligation is run on a gel, three bands will be obtained. The target band will be of the order of 1.5kbases in length.

This scheme is illustrated diagrammatically in Figure 1 hereinafter

The ligation obtained in this way, or the product of the second PCR reaction forms the deleted locus which can then be used to form a suitable deletion plasmid. For example, the deleted locus may be cut with a restriction enzyme such as *XbaI* and cloned into pUC18. Suitable hosts such as *E. coli* DH5α can be used as the host for the initial cloning although TG90, which replicates high-copy number plasmids at low copy number, may be useful in some instances.

This is then subcloned into a suitable plasmid to form a deletion plasmid.

A particularly suitable plasmid is pOR120 (see Figure 2), which has been shown to work effectively in *B. subtilis (Leehouts et al, 1996, Mol. Gen. Genet. 253:217-224)* and will allow unlabelled gene deletions or replacements to be made in bacterial chromosomes. It is derived from the lactococcal plasmid pWV)1, which lacks the *repA* gene (replication initiation protein), so will only replicate in strains expressing PepA in trans, such as E. coli EC1000. It contains the tetracycline resistance gene (*tet*) to enable selection of recombinants and the β-galactosidase gene (*lacZ*) to allow identification of revertants by blue/white selection. The lacZ is under the control of a p32 promoter (allowing expression in both gram-positive and gram-negative bacteria).

The deletion locus can then be subcloned into the pORI240 plasmid and transformants selected in a suitable host strain such as *E. coli* EC1000 by tetracycline resistance. Strains of *B. subtilis* and preferably strains of known provenance such as *B. subtilis* 168 can then be transformed using this plasmids, and transformants selected on tetracycline. (*B. subtilis* 168 is the strain used in the complete genome sequencing project [see Kunst et al. supra.] and can be obtained from the Institut Pasteur (CIP no. 106309). However, *Bacillus subtilis* WB600 may also be improved using this method.

The plasmid is unable to replicate in this strain, so transformants will have undergone chromosomal integration.

Then a second recombination event (resolving the cointegrant) which will yield white colonies with X-gal present (integrants will remain blue) can be readily selected.

Figure 2 illustrates the strategy for gene deletion using pORI240, where the * symbol represents the deletion of the target gene (adapted from Leenhouts et al.). Using this method, either the wild type gene is restored, or the deleted copy is inserted in its place upon resolution of the integrated plasmid.

Revertant clones can be screened out genotypically by PCR to confirm the deletion. Loss of protease activity may be confirmed phenotypically using Zymogram gele analysis (Novex) to confirm the loss of protease activity.

Other strategies for producing gene deletions can be employed.

Once an asporogenic strain, which is also protease deficient, has been prepared, it can be transformed using conventional methods, so that it expresses a gene of choice, and in particular a heterologous gene. Although particularly suited to the expression of antigens or proteins useful as vaccines such as PA of *B. anthracis* as described above,or immunogenic fragments or domains or variants thereof, it can be used as a expression vehicle for a wide range of proteins. It may be particularly useful where the more conventional expression hosts such as *E. coli,* are unsuited to expression of a particular target gene for example for reasons of toxicity of the protein to the *E. coli*.

Thus in a further aspect, the invention provides a recombinant asporogenic strain of *B. subtilis* as described above, which has

been transformed such that it contains a heterologous gene arranged such that the gene expressed.

As mentioned above, the heterologous gene may encode a wide range of desired proteins, for example pharmaceutical or agrochemical proteins or peptides, or other proteins which have commercial applications such as enzymes, for example cellulases and amylases, used in paper manufacture or detergent manufacture. One particular form or pharmaceutical protein will be immunogenic proteins or peptides such as antigens, intended for use as a vaccine. Another form may be hormones such as human growth hormone.

A particularly preferred heterologous gene encodes PA of *B. anthracis* as described above, or immunogenic fragments, or a variant of any of these, which produce a protective immune response.

As used herein, the term "variant" refers to sequences of amino acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 60% identical, preferably at least 75% identical, and more preferably at least 90% identical to the base sequence.

In particular, the identity of a particular sequence to the sequence on which they are based may be assessed using the multiple alignment method described by Lipman and Pearson, (Lipman, D.J. & Pearson, W.R. (1985) Rapid and Sensitive Protein

Similarity Searches, Science, vol 227, pp1435-1441). The "optimised" percentage score should be calculated with the following parameters for the Lipman-Pearson algorithm:ktup =1, gap penalty =4 and gap penalty length =12. The sequences for which similarity is to be assessed should be used as the "test sequence" which means that the base sequence for the comparison, such as the sequence of PA of *B. anthracis* should be entered first into the algorithm.

The term "fragment" as used herein refers to truncated regions which lack one of more amino acids as compared to the full length sequence, but which produce a protective immune response. They may comprise domains.

Particular protective domains of the PA of *B. anthracis* comprise domains 1 or 4 of the full length sequence, or protective regions of these domains. These domains comprise the following sequences shown in the following Table 1.

Table 1

Domain	Amino acids of full-length PA*
4	596-736
1	1-258

These amino acids numbers refer to the sequence as shown in Welkos et al. Gene 69 (1988) 287-300. Domain 1 comprises two regions, designated 1a and 1b. Region 1a comprises amino acids 1-169 whereas region 1b is from amino acid 170-258. It appears that region 1a is important for the production of a good protective response.

In a particularly preferred embodiment, a combination of domains 1 and 4, or protective regions thereof, are used as the immunogenic reagent which gives rise to an immune response protective against *B. anthracis*. This combination, for example

as a fusion peptide, may be expressed using the expression system of the invention.

In a further aspect, the invention comprises a method for producing a target protein, said method comprising transforming a recombinant asporogenic strain of *B. subtilis* as described above which a nucleotide sequence which encodes said protein, culturing said transformed strain and recovering said target protein from the culture.

Conventional methods, such as are generally used for the transformation and culture of microorganisms are suitably employed.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which

Figure 1 illustrates a strategy for producing a locus for use in gene deletion; and

Figure 2 illustrates the strategy for gene deletion using pORI240.

Example 1

Construction of a protease-deficient asporogenic strain of *Bacillus subtilis* 168

The gene encoding the sigma factor, *spoIIAC,* was amplified using PCR, together with upstream flanking DNA encoding both *spoIIAA* and *spoIIAB* and downstream flanking DNA. The resultant 2.663kb segment of DNA was synthesised by the method of Higuchi et al., Nucl. Acids Res. (1988), 16, 7351, to contain a *XhoI* restriction endonuclease site. This site was located 460bp into the 760bp *spoIIAC* gene. The PCR fragment was cloned into the general cloning vector pGEM3Zf (Promega) creating the plasmid pAUS-Y.

Two subsequent plasmids were constructed from the pAUS-Y by digestion and insertion of an erythromycin resistance cassette (*erm*) from the plasmid pDG646 (Guérot-Fleury et al., 1995. Gene, 167: 335). The first of these plasmids, pDINGO, was made by digesting pAUS-Y with *XhoI* and inserting a *XhoI* digested *erm* fragment. The second plasmid, pREEF, was made by digesting pAUS-Y with *XHoI* and *ClAI* to remove the last 90 bp of *spoIIAB* and the first 460bp of *spoIIAC*. A *SalI/ClaI* digested *erm* fragment was inserted.

Both plasmids were linearised by digestion with *ScaI* and the DNA was then used to transform competant cells of *B. subtilis* WB600. Transformed clones were selected with erythromycin/lincomycin and genomic DNA isolated from these clones was analysed by Southern hybridisation. Digoxigenein-labelled DNA probes from the *spoIIAC::erm* locus were used to identify clones which contained an *erm* containing allelic replacement of *spoIIAC*.

The insertion of DNA into genes using this technique has been found to be stable and non-reverting. No reversion, (measured by the absence of spore formation) was encountered with the *spoIIAC::erm* strains.

Example 2

Production of Protease Deficient strains of B. subtilis

A series of primers suitable from the construction of a protease-deficient asporogenic strain of *Bacillus subtilis* 168, using the method outlined in Figure 1.

Specifically, the primers were designed to amplify the 5′ and 3′ regions flanking the gene to be deleted. The external primers (5XBA and 3XBA) are 25-26 nucleotides and incorporate an XbaI site at the 5′ end. The internal primers (5XHO and 3XHO) are 41-43 nucleotides in length with target site homology on their 3′ end only, then a XhoI site, then a 5′ end with homology to

the other primer, not to the target site. The 24 protease gene deletion primers are shown below 5'-3'.

APRE5XBA (25nt);
GCTTCTAGATGAAGCCAATATTCCG (SEQ ID NO 1)

APRE5XHO (43nt)
TATACCTAAATAGAGCTCCATACCTGCTTCTTTTATTTGTCAG (SEQ ID NO 2)

APRE3XHO (41nt)
GAAGCAGGTATGGAGCTCTATTTAGGTATATCATCTCTCGC (SEQ ID NO 3)

APRE3XBA (25nt)
AAGTCTAGAAATAACGTTGACATTC (SEQ ID NO 4)

BPR5XBA (25nt)
TCCTCTAGAAACATCACTGGAGGAC (SEQ ID NO 5)

BPR5XHO (42nt)
TCTGCTTAATTTCTCGAGAACATCGCTGTATATTAACTGTAG (SEQ ID NO 6)

BPR3XHO (42 nt)
TACTGCGATGTTCTCGAGAAATTAAGCAGATTTCCCTGAAAA (SEQ ID NO 7)

BPR3XBA (25 nt)
GCTTCTAGATGGCTTCCAATGGGTC (SEQ ID NO 8)

EPR5XBA (26 nt)
ACCTCTAGATTTCGGTTGAAAACAAG (SEQ ID NO 9)

EPR5XHO (43 nt)
AGTATGAAAAGCCTCGAGGAAATTTTCCAAATGAATTTGTAAG (SEQ ID NO 10)

EPR3XHO (42 nt)
TTGGAAAATTTCCTCGAGGCTTTTCATACTATTGCTATACAG (SEQ ID NO 11)

EPR3XBA (25 nt)
TGTTCTAGACACGAGGTCGAGCATT (SEQ ID NO 12)

MPR5XBA (26 nt)
ATTTCTAGAGCCGATCGGTCATGTGC (SEQ ID NO 13)

MPR5XHO (41 nt)
CCCCTTAGCATCCTCGAGGTTTCTGATTCTTATGATAAAAC (SEQ ID NO 14)

MPR3XHO (43 nt)
AGAATCAGAAACCTCGAGGATGCTAAGGGGCTGCCGGTCGAAG (SEQ ID NO 15)

MPR3XBA (25 nt)
CCGTCTAGATGTGCCGTCCAAGTCC (SEQ ID NO 16)

NPRB5XBA (26 nt)
GACTCTAGACATCTGCCGCTGGCTTG (SEQ ID NO 17)

NPRB5XHO (43 nt)
TGTTTTTGTTTGCTCGAGATATTTCGCTCTGCCCTTCTTTTTC (SEQ ID NO 18)

NPRB3XHO (42 nt)
AGAGCGAAATATCTCGAGCAAACAAAAACAGTCAGGACACAG (SEQ ID NO 19)

NPRB3XBA (25 nt)
CGATCTAGAATTCGAAGATGCAGTC (SEQ ID NO 20)

NPRE5XBA (25 nt)
GAATCTAGAAAGTATCCAGTCCCGC (SEQ ID NO 21)

NPRE5XHO (41 nt)
TTTTGTTGAGTACTCGAGACAGAAAACCGCTCCTGATTTGC (SEQ ID NO 22)

NPRE3XHO (41 nt)
GCGGTTTTCTGTCTCGAGTACTCAACAAAAACTAACATAAC (SEQ ID NO 23)

NPRE3XBA (26 nt)
GCATCTAGACAAGCAGTCCGATAATC (SEQ ID NO 24)

PCR followed by ligation or treatment as outlined above and summarised in Figures 1 and 2, using some or all of the above sets of primers will result in a protease deficient strain of *B. subtilis*.

The process can either be repeated with appropriate primers to delete the spoIIAC gene, or the strategy outlined in Example 1 can be repeated to yield an asporogenic strain.

The deletions can be carried out in any order.

Claims

1. A recombinant microorganism comprising an asporogenic strain of *Bacillus subtilis* in which at least one gene which encodes a protease enzyme has been downregulated or inactivated.

2. A recombinant microorganism according to claim 1 wherein said protease enzyme is serine alkaline protease E (*aprE*), bacillopeptidase F (*bpr*), extracellular serine protease (*epr*), extracellular metalloprotease (*mpr*), extracellular neutral protease (*nprB*) or extracellular neutral metalloprotease (*nprE*).

3. A recombinant microorganism according to claim 2 wherein all of the said protease enzyme genes are inactivated.

4. A recombinant microrganism according to any one of the preceding claims wherein the said protease enzyme gene is deleted.

5. A recombinant microorganism according to any one of the preceding claims wherein a gene encoding sigma factor *spoIIAC* has been inactivated such that the strain is asporogenic.

6. A recombinant microorganism according to claim 5 wherein the gene encoding sigma factor *spoIIAC* is partially or totally deleted, and/or been subject to insertion mutagenesis.

7. A recombinant microorganism according to any one of the preceding claims which comprises a mutated form of *B. subtilis* 168.

8. A recombinant microorganism according to any one of the preceding claims, which has been transformed such that it contains a heterologous gene arranged such that the gene is expressed.

9. A recombinant microorganism according to claim 8 wherein said heterologous gene encodes an antigens or proteins useful in the production of a protective immune response to a pathogen.

10. A recombinant microorganism according to claim 9 wherein said heterologous gene encodes PA of *B. anthracis* or an immunogenic fragments or domains thereof, or a variant of any of these.

11. A recombinant microorganism according to claim 10 wherein said heterologous gene encodes PA of *B. anthracis* or one or more of domains 1 and 4 or protective regions thereof, of the full length sequence.

12. A method for producing a target protein, said method comprising transforming a recombinant microorganism according to any one of claims 1 to 11 with a nucleotide sequence which encodes said protein, culturing said transformed strain and recovering said target protein from the culture.

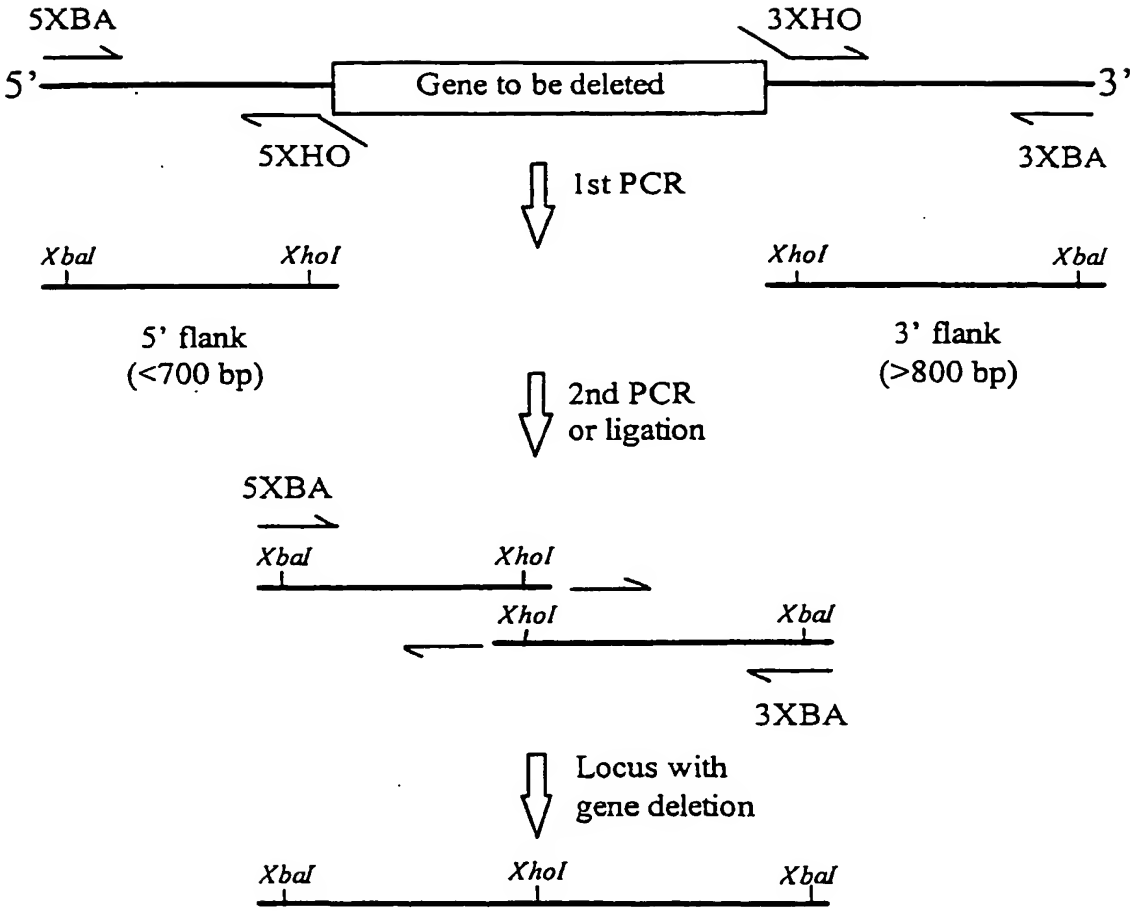
5XBA
3XHO
5'
Gene to be deleted
3'
5XHO
3XBA
1st PCR
XbaI
XhoI
XhoI
XbaI
5' flank
(<700 bp)
3' flank
(>800 bp)
2nd PCR
or ligation
5XBA
XbaI
XhoI
XhoI
XbaI
3XBA
Locus with
gene deletion
XbaI
XhoI
XbaI

Figure 1

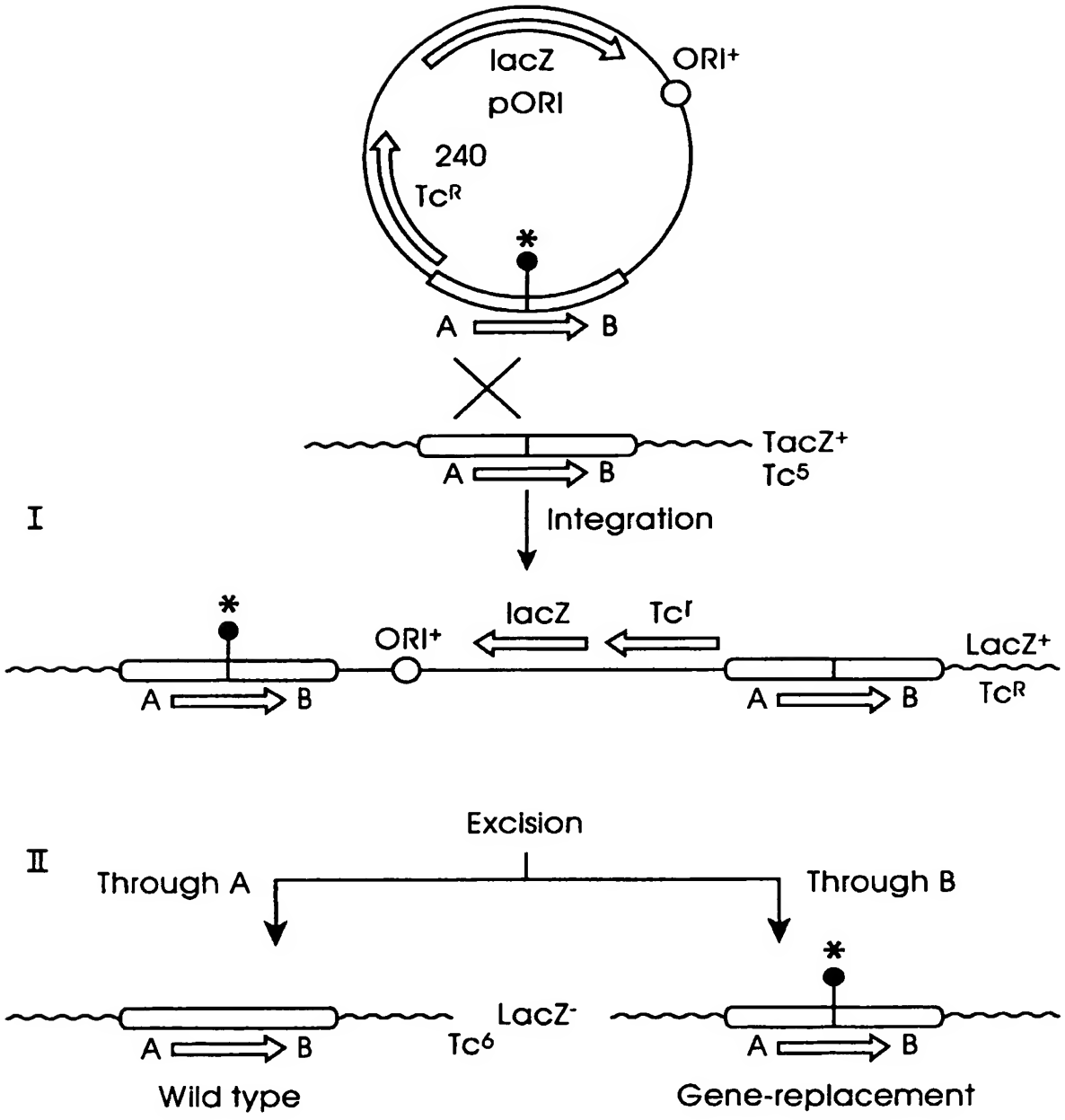
lacZ
pORI
ORI+
240
TcR
*
A
B
TacZ+
Tc5
I
Integration
lacZ
Tcr
ORI+
LacZ+
TcR
II
Excision
Through A
Through B
LacZ-
Tc6
Wild type
Gene-replacement

Fig. 2